

Biologic and genetic characteristics of *Toxoplasma gondii* isolates in free-range chickens from Nicaragua, Central America

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Abstract

The prevalence of *Toxoplasma gondii* in free-ranging chickens is a good indicator of the prevalence of *T. gondii* oocysts in the soil because chickens feed from the ground. The prevalence of *T. gondii* in 98 free-range chickens (*Gallus domesticus*) from Nicaragua was determined. Antibodies to *T. gondii* were assayed by the modified agglutination test (MAT), and found in 84 (85.7%) of 98 chickens with titers of 1:5 in 10, 1:10 in eight, 1:20 in seven, 1:40 in nine, 1:80 in 11, 1:160 in one, 1:200 in 27, 1:400 in six, 1:800 four, and 1:3200 in one bird. Hearts and brains of 32 chickens with titers of 1:10 or less were pooled and fed to three *T. gondii*-free cats. Hearts and brains of 66 chickens with titers of 1:20 or higher were bioassayed in mice. Feces of cats were examined for oocysts. The cat fed tissues from eight chickens with titers of 1:10 shed *T. gondii* oocysts. The two cats fed tissues of 24 chickens with titers of 1:5 or less did not shed oocysts. *T. gondii* was isolated by bioassay in mice from 47 chickens with MAT titers of 1:20 or higher. All infected mice from six isolates died of toxoplasmosis. Overall, 41 of 170 (24.1%) mice that became infected after inoculation with chicken tissues died of toxoplasmosis. Genotyping of these 48 isolates (47 from mice and 1 from pooled tissues) using polymorphisms at the loci SAG1, SAG2, SAG3, BTUB and GRA6 revealed eight genotypes. Six isolates had Type I alleles, three isolate had Type II alleles and six isolates had Type III alleles at all loci. Four isolates had mixed infections. Two isolates have a unique allele at SAG1 locus and combination of I and III alleles at other loci. The rest 27 isolates contained the combination of Type I and III alleles and were divided into four genotypes. More than one genotypes were often isolated in chickens from the same household, indicating multiple genotypes were circulating in the same environment. This may explain the high frequency of mixed infections observed. High rate of mixed infection in intermediate hosts such as chickens may facilitate genetic exchange between different parasite lineages in definitive feline hosts. This is the first report of genetic characterization of *T. gondii* isolates from Nicaragua, Central America.

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Keywords: *Toxoplasma gondii*; Chickens; *Gallus domesticus*; Free-range; Nicaragua; Central America; Genotype

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1. Introduction

Toxoplasma gondii infections are widely prevalent in human beings and animals worldwide (Dubey and Beattie, 1988). Humans become infected post-natally by ingesting tissue cysts from undercooked meat, consuming food or drink contaminated with oocysts, or by accidentally ingesting oocysts from the environment. However, only a small percentage of exposed adult humans develop clinical signs. It is unknown whether the severity of toxoplasmosis in immunocompetent persons is due to the parasite strain, host variability, or to other factors.

T. gondii isolates have been classified into three genetic types (I, II, III) based on restriction fragment length polymorphism (RFLP) (Ajzenberg et al., 2002a,b, 2004; Aspinall et al., 2003; Boothroyd and Grigg, 2002; da Silva et al., 2005; Dubey et al., 2004a,d; Ferreira et al., 2004, 2006; Fuentes et al., 2001; Grigg et al., 2001; Howe and Sibley, 1995; Howe et al., 1997; Jungersen et al., 2002; Mondragon et al., 1998; Owen and Trees, 1999). The parasite was previously considered clonal with very low genetic variability. However, most of the information was derived from isolates from Europe and North America. Using newer markers for genetic characterization and using recently isolated strains from Brazil and French Guyana, higher genetic variability was revealed than previously reported (Ajzenberg et al., 2004; Lehmann et al., 2004).

We have initiated a worldwide study of *T. gondii* population structure. For this we have chosen the free-range chicken as the indicator host for soil contamination with *T. gondii* oocysts because they feed from the ground (Ruiz and Frenkel, 1980). Thus far, we have characterized strains from South America (Brazil [Dubey et al., 2002; Dubey et al., 2003a; Dubey et al., 2003d; Dubey et al., 2006a], Peru [Dubey et al., 2004b], Venezuela [Dubey et al., 2005h], Argentina [Dubey et al., 2003e; Dubey et al., 2005f]), Colombia [Dubey et al., 2005d], Chile [Dubey et al., in press-a]; Central America and the Caribbean (Guatemala [Dubey et al., 2005e], Grenada, West Indies [Dubey et al., 2005b], Costa Rica [Dubey et al., 2006c], North America (USA [Dubey et al., 2003c; Lehmann et al., 2003], Mexico [Dubey et al., 2004c]), Africa and Middle East (Egypt [Dubey et al., 2003b], Israel [Dubey et al., 2004e], Mali, Kenya, Burkina Faso, and Democratic Republic of Congo [Dubey et al., 2005a]), Asia (Sri Lanka [Dubey et al., 2005g], India [Sreekumar et al., 2003]), Europe (Austria [Dubey et al., 2005c], and Portugal [Dubey et al., 2005, 2006b]). These studies are still not complete, nevertheless, a

pattern is emerging that isolates from Brazil are genetically distinct (Lehmann et al., 2004).

Before the recognition of three genotypes of *T. gondii* (Howe and Sibley, 1995), *T. gondii* isolates were phenotypically classified as mouse virulent or avirulent. Type I strains were considered mouse virulent whereas Type II and Type III strains were avirulent or mildly virulent for mice (Howe and Sibley, 1995); Type I strains killed all mice within 2 week post-inoculation (p.i.), irrespective of the dose. However, these data are based on isolates that have been maintained in mice for an unknown time (Howe and Sibley, 1995). There are very few data on mouse mortality based on primary isolations. We have started to accumulate such data based on isolates from chickens using a specified protocol (subcutaneous inoculation of tissue digest into four to five SW mice).

In the present paper, we report on biologic and genetic characteristics of *T. gondii* isolates from chickens from Nicaragua, Central America.

2. Materials and methods

2.1. Naturally-infected chickens

In Nicaragua smallholder poultry production is wide-spread. Thus, 71% of 199,549 rural households kept 1,269,116 adult hens, 241,296 roosters, and 1,479,204 replacements in the most recent agricultural census (CENAGRO, 2002). These chickens are kept free-range without fencing and only housed at night. For the present study, samples ($n = 98$) were obtained from free-range chickens from the El Sauce municipality (Fig. 1) within a radius of 10–15 km in different directions from El Sauce town (latitude 12°53'13N and longitude 86°32'17 W). The chickens originated from 36 different households that were at least 500 m apart. Eighteen households provided one chicken, six provided two, three provided three, one provided four, two provided five, two provided six, three provided eight and one household provided eleven chickens (Table 1).

Chickens were purchased, identified and housed together until they were killed on 28 November 2005. Samples of brain, whole heart, and blood were collected from each chicken, and kept at 4 °C until sent with cold packs by air to Beltsville, MD. Two days elapsed between killing of chickens and receipt of samples at Beltsville. Samples were received in excellent condition.

2.2. Serological examination

Sera of chickens were tested for *T. gondii* antibodies using eight dilutions, from 1:5 to 1: 640 with the



Fig. 1. Map of Nicaragua showing El Sauce municipality.

modified agglutination test (MAT) as described by Dubey and Desmonts (1987).

2.3. Bioassay of chickens for *T. gondii* infection

Tissues of all chickens were bioassayed for *T. gondii* infection. Brains, and hearts of 66 chickens with titers of 1:20 or higher were bioassayed individually in outbred female Swiss Webster (SW) mice obtained from Taconic Farms, Germantown, New York, as described (Dubey et al., 2002). Tissues were homogenized, digested in acidic pepsin, washed, and homogenate inoculated subcutaneously into four mice (Dubey, 1998).

Brains and hearts from 32 chickens with MAT titers of <1:20 were pooled and fed to three *T. gondii*-free cats (14 chickens < 1:5 to one cat, 10 chickens 1:5 to one cat, and eight chickens 1:10 to one cat, Dubey et al., 2002). Feces of cats were examined for shedding of *T. gondii* oocysts 3–14 days post-ingesting chicken tissues as previously described (Dubey, 1995). Fecal floats were incubated in 2% sulfuric acid for 1 week at room temperature on a shaker to allow sporulation of oocysts and were bioassayed orally in mice (Dubey and Beattie, 1988). Tissue imprints of lungs and brains of mice that died were examined for *T. gondii* tachyzoites or tissue cysts. Survivors were bled on day 41 p.i. and a 1:25 dilution of serum from each mouse was tested for *T. gondii* antibodies with the MAT. Mice were killed 42 days p.i. and brains of all mice were examined for tissue cysts as described (Dubey and Beattie, 1988). The

inoculated mice were considered infected with *T. gondii* when tachyzoites or tissue cysts were found in tissues.

2.4. Genetic characterization for *T. gondii*

T. gondii DNA was extracted from the tissues of all infected mice from each group (Table 1) and strain typing was performed using genetic markers SAG1, SAG2, SAG3, BTUB and GRA6 as described with modification (Grigg et al., 2001; Howe et al., 1997; Khan et al., 2005). In brief, the target DNA sequences were first amplified by multiplex PCR using external primers for all five markers. The reaction was carried out in 25 µl of volume containing 1× PCR buffer, 2 mM MgCl₂, 200 µM each of the dNTPs, 0.15 µM each of the forward and reverse primers, 0.5 units of FastStart DNA polymerase and 1.5 µl of DNA extract. The reaction mixture was treated at 95 °C for 4 min, followed by 25 cycles of 94 °C for 30 min, 55 °C for 1 min and 72 °C for 2 min. Multiplex PCR amplified products (1.5 µl) were then used for second round amplification (35 cycles) with internal primers for each marker separately, using an annealing temperature of 60 °C in 25 µl volume reaction mixture. To reveal the RFLP pattern of each reference strain, 3 µl of PCR products were mixed with 17 µl of digestion reaction containing 1× NEB buffer, 0.1 mg/ml BSA and 1 unit of restriction enzyme. The reaction was carried out by incubating at the proper temperature for each restriction enzyme by the manufacturer's instruction (New England BioLab, Beverly, MA). The digested PCR products were resolved in a 2.5–3% agarose gel by

Table 1
Isolation of *T. gondii* from free-range chickens from Niaragua

Chickens			Isolation in mice			Genotype ^y					
No.	Farmhold location ^{a-w}	MAT titer	No. infected ^x	No. died	Day of death	Isolate ID	SAG1	SAG2	SAG3	BTUB	GRA6
1	Tololos ^u	200	4	2	17, 39	TgCkNi1	I (4)	I (4)	III (4)	III (4)	I (4)
5	La Suiza (Tololos) ^r	200	4	1	21	TgCkNi2	I & II or III (2) II or III (2)	III (4)	III (4)	I & III (3), III (1)	III (4)
8	San Nicolas (Tololos) ^h	20	4	0	n/a	TgCkNi3	II or III (4)	III (4)	III (4)	III (4)	III (4)
9	La Suiza (Tololos) ^r	400	4	4	12, 14, 18, 23	TgCkNi4	I (4)	I (4)	III (4)	I (4)	III (4)
13	Santa Teresa (Salitre) ^p	200	4	1	17	TgCkNi5	I (4)	I (4)	III (4)	I (4)	III (4)
14	Tololos ⁱ	400	4	2	19, 19	TgCkNi6	I (4)	I (4)	III (4)	I (4)	III (4)
17	Panales ^v	200	4	1	23	TgCkNi7	II or III (1), I (3)	I (3), III (1)	I (4)	I (3), III (1)	I (4)
18	Tololos ^u	80	4	0	n/a	TgCkNi8	II or III (4)	III (4)	III (4)	III (4)	III (4)
19	Palma ^o	800	4	4	7, 11, 11, 11	TgCkNi9	I (4)	I (4)	I (4)	I (4)	I (4)
21	San Nicolas (Tololos) ^m	200	1	0	n/a	TgCkNi10	I (1)	I (1)	III (1)	I (1)	III (1)
24	Tololos ^d	200	3	0	n/a	TgCkNi11	I (3)	I (3)	III (3)	III (3)	I (3)
27	Santa Teresa (Salitre) ^a	800	4	1	23	TgCkNi12	u-1 (4)	I (4)	III (4)	I (4)	III (4)
30	El Sauce ^g	80	4	0	n/a	TgCkNi13	II or III (3)	III (3)	III (3)	III (3)	III (3)
32	Tololos ^d	200	4	1	13	TgCkNi14	I (4)	III (4)	III (4)	III (4)	III (4)
33	Valle San Antonio ^b	40	4	0	n/a	TgCkNi15	I (4)	III (4)	III (4)	III (4)	III (4)
35	Santa Cruz (Panales) ^t	20	4	0	n/a	TgCkNi16	II or III (4)	III (4)	I (4)	III (4)	I (4)
36	Santa Teresa (Salitre) ^a	≥3200	4	0	n/a	TgCkNi17	I (4)	I (4)	III (4)	I (3)	III (3)
39	San Nicolas (Tololos) ^m	20	3	0	n/a	TgCkNi18	II or III (3)	II (3)	II (3)	II (3)	II (3)
40	Panales ^l	200	3	1	46	TgCkNi19	I (3)	I (3)	III (3)	III (3)	I (3)
41	Tololos ^d	40	3	0	n/a	TgCkNi20	I (3)	III (3)	III (3)	III (3)	III (3)
42	Tololos ^d	200	2	0	n/a	TgCkNi21	I (2)	I (2)	III (2)	I (2)	III (2)
43	Tololos ^d	40	4	0	n/a	TgCkNi22	I (4)	I (4)	III (4)	III (4)	I (4)
49	Tololos ^d	200	4	0	n/a	TgCkNi23	I (4)	I (4)	III (4)	III (3)	I (4)
51	El España (Panales) ^q	200	2	0	n/a	TgCkNi24	I (2)	I (2)	III (2)	III (2)	I (2)
52	Tololos ⁱ	200	4	0	n/a	TgCkNi25	I (4)	III (4)	III (4)	III (4)	III (4)
53	Valle San Antonio ^c	200	4	0	n/a	TgCkNi26	I (4)	I (4)	III (4)	III (4)	I (4)
56	Laguneta (Panales) ^j	80	3	0	n/a	TgCkNi27	II or III (3)	III (2)	III (3)	III (4)	III (4)
58	San Nicolas (Tololos) ⁿ	200	3	2	30, 37	TgCkNi28	I (3)	I (3)	I (3)	I (3)	I (3)
59	El España (Panales) ^q	200	4	0	n/a	TgCkNi29	I (4)	I (4)	III (4)	III (4)	I (4)
60	Valle San Antonio ^e	40	4	0	n/a	TgCkNi30	I (4)	III (4)	III (4)	III (4)	III (4)
61	Panales 2 ^v	200	4	4	16, 17, 17, 19	TgCkNi31	I (4)	I (4)	I (4)	I (4)	I (4)
62	El Sauce ^g	200	4	0	n/a	TgCkNi32	u-1 (4)	I (4)	III (4)	I (4)	III (4)
63	El España (Panales) ^s	400	4	0	n/a	TgCkNi33	I (4)	I (4)	III (4)	III (4)	I (4)
64	Estación (El Sauce) ^f	400	4	0	n/a	TgCkNi34	I (4)	I (4)	III (4)	I (4)	III (4)
65	San Nicolas (Tololos) ^r	800	4	1	20	TgCkNi35	I (4)	I (4)	III (4)	I (3)	III (4)
67	Panales ^v	200	2	0	n/a	TgCkNi36	I (2)	I (2)	III (2)	III (2)	I (2)
70	San Nicolas (Tololos) ^k	80	4	0	n/a	TgCkNi37	I (4)	I (3)	III (4)	I (4)	III (4)
72	Tololos ⁱ	20	2	0	n/a	TgCkNi38	I (2)	I (2)	III (2)	III (1)	I (2)
73	San Nicolas ⁿ	800	4	0	n/a	TgCkNi39	II or III (4)	II (4)	II (4)	II (4)	II (4)

75	Panales 2 ^v	200	4	4	13, 14, 14, 16	TgCkNi40	I (4)	I (4)	I (4)	I (4)
77	Pavon	20	3	3	21, 23, 27	TgCkNi 41	I (3)	I (3)	I (3)	I (3)
84	Santa Teresa (Salitre) ^w	200	4	1	40	TgCkNi42	II or III (4)	II (4)	II (4)	II (4)
85	Panales 2 ⁱ	200	4	4	14, 15, 15, 19	TgCkNi43	I (4)	I (4)	I (4)	I (4)
92	El Sauce ^g	≥640	4	3	13, 14, 21	TgCkNi44	II or III (4)	III (4)	III (3)	III (3)
94	Santa Cruz (Panales) ^t	160	4	0	n/a	TgCkNi45	II or III (4)	III (4)	III (4)	I (4)
97	Tololos ⁱ	80	4	0	n/a	TgCkNi46	I (4)	III (4)	III (3), I & III (1)	I (3), I & III (1)
98	Panales ⁱ	80	4	1	27	TgCkNi47	I (4)	I (2), III (2)	I & III (1), I (2), III (1)	I (4)
Pooled tissues		10	Not applicable			TgCkNi48	II or III	III	III	III

(x) Of four mice inoculated. (a-w) Households designated by different letters. (y) Genotyping based on DNA from the stated no. of mice. (u-1) is an unique allele identified at SAG1 locus. n/a = not applicable.

electrophoresis in the presence of 0.3 µg/ml ethidium bromide and visualized under UV light. The primers and enzymes used were stated previously (Dubey et al., in press-b).

3. Results

Antibodies to *T. gondii* were found in 84 (85.7%) of 98 chickens with titers of 1:5 in 10, 1:10 in eight, 1:20 in seven, 1:40 in nine, 1:80 in 11, 1:160 in one, 1:200 in 27, 1:400 in six, 1:800 four, and 1:3200 in one bird.

T. gondii was isolated by bioassay in mice from 47 chickens with MAT titers of 1:20 or higher. All infected mice from six isolates died of toxoplasmosis. Overall, 41 of 170 (24.1%) mice that became infected after inoculation with chicken tissues died of toxoplasmosis. All mice that became infected after inoculation with tissues from six chicken (9, 19, 61, 75, 77, and 85) died of acute toxoplasmosis between 6 and 27 days p.i.

Genotyping of these 47 isolates using polymorphisms at the loci SAG1, SAG2, SAG3, BTUB and GRA6 revealed eight genotypes. Six isolates (TgCkNi9, 28, 31, 40, 41, 43) had Type I alleles, three isolate (TgCkNi18, 39, 42) had Type II at all loci, and five isolates (TgCkNi3, 8, 13, 27, 44) had Type III alleles at all loci. Two isolates (TgCkNi12, 32) have a unique allele at SAG1 locus and combination of I and III alleles at other loci. The rest 27 isolates contained the combination of Type I and III alleles and were divided into four genotypes. Of these 27 isolates, 11 isolates (TgCkNi1, 11, 19, 22, 23, 24, 26, 29, 33, 36, 38) have I, I, III, III and I alleles, nine isolates (TgCkNi4, 5, 6, 10, 17, 21, 34, 35, 37) have I, I, III, I and III alleles, two isolates (TgCkNi16, 45) have II or III, III, I, III and I alleles, five isolates (TgCkNi14, 15, 20, 25, 30) have I, III, III, III and III alleles at loci SAG1, SAG2, SAG3, BTUB and GRA6, respectively. Four isolates (TgCkNi2, 7, 46, 47) had mixed infections.

The cat (no. 230) fed pooled tissues from eight chickens with titers of 1:10 shed *T. gondii* oocysts. The two mice fed oocysts from cat 230 died of acute toxoplasmosis 4 days later and numerous tachyzoites were found in their mesenteric lymph nodes; these tachyzoites were infective to mice by the subcutaneous route. Genotyping of this isolate (TgCkNi48) revealed the Type III alleles at all loci.

The two cats fed tissues of 24 chickens with titers of 1:5 or less did not shed oocysts.

4. Discussion

It is interesting to see that multiple genotypes were identified in chickens from the same household. From

one household in Tololos, six isolates (TgCkNi11, 14, 20, 21, 22, 23) were typed into three genotypes. Three isolates (TgCkNi18, 28, 39) from a household in San Nicolas were typed into two genotypes. Similar phenomenon was observed in a few other locations. This clearly indicate that more than one genotype are circulating in a given area in El Sauce municipality. This may explain the high frequency of mixed infections observed. High rate of mixed infection in intermediate hosts (such as chickens) will likely lead to more frequent genetic exchange between different parasite lineages when the intermediate hosts are preyed by feline hosts, which in turn will facilitate the evolution of *T. gondii*.

Phenotypically and genetically, *T. gondii* isolates from chickens from Nicaragua were different from the isolates from North America and Grenada, West Indies but similar to those from Costa Rica. Most isolates from chickens from Brazil and Colombia were lethal for mice whereas isolates from North America and the Caribbean did not kill inoculated mice. Genetically, none of *T. gondii* isolates from Colombia and Brazil was SAG2 Type II, whereas most isolates from chickens from North America and Grenada were Type II (Dubey et al., 2003c; Lehmann et al., 2003). This is the first report of genetic characterization of *T. gondii* isolates from Nicaragua.

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